REMARKS

Claims 132-146, 148-154 and 171-176 are pending in the application. Claims 148-154 have been withdrawn. Claims 133-134, 138-139 and 143-144 have been objected to. Claims 132-146 and 171-176 have been rejected under 35 USC 112. Claims 132-146 and 171-176 have been rejected under 35 USC 102 as being anticipated by Wang et al (2001), Leung et al. (1996) and Wang et al (US 20050074763). Claims 132-146 and 171-172 have been amended. Claims 173-176 have been canceled. Support for the amended claims may be found throughout the instant specification, as detailed below. Thus, no new subject material has been introduced.

Priority

The Examiner has stated that the benefit date of priority is September 4, 2002. However, review of USPTO records indicates that the instant application is a National Phase application of PCT IL03/00723, filed September 2, 2003, which is an International Filing of US Patent Application No. 10/233,409, filed September 4, 2002, which in turn is a Continuation-In-Part of PCT/IL02/00174, filed March 5, 2002, which claims priority from U.S. Provisional Patent Application No. 60/272,771, filed March 5, 2001. Inasmuch as the instant specification includes all of the disclosures of both PCT IL03/00723 and PCT IL02/00174, Applicants submit that the instant application qualifies for a benefit date of priority of March 5, 2001.

The instant specification (see *supra*) has been amended to show related applications and priority. Petition for unintentional delayed claim of priority, according to 37 USC 1.78(a)(3) is being filed, along with requisite fees.

Claims Objections

The Examiner has objected to claims 133-134, 138-139 and 143-144 for reciting non-elected SEQ ID NOs. Claims 133-134, 138-139 and 143-144 have now been amended to recite only SEQ ID NOs: 1 or 2.

Claims Rejections: 35 U.S.C. § 112 1st Paragraph

The Examiner has rejected claims 133-134, 138-139 and 143-144 under 35 U.S.C. 112 1st paragraph for lacking enablement and adequate written description. The Examiner's rejections are respectfully traversed. Claims 132-146 have now been amended.

The Examiner has asserted that while the specification is enabling for the boiling-stable, detergent stable and protease resistant polypeptides as encoded by a polynucleotide as set forth in SEQ ID NO:1, or having an amino acid sequence as set forth in SEQ ID NO:2, the specification lacks enablement for polynucleotides and/or polypeptides being homologues of the indicated SEQ IDs. The Examiner has based the rejection on the determination that

> "The amount of guidance is zero with regards to which amino acids in instant SEQ ID NO:2 are essential for activity. No working examples are present [in the specification] of homologs of instant SEQ ID NO:2".(page 4 of instant communication)

Applicant strongly disagrees.

Applicant wishes to point out that the claimed stress-related, boiling and detergent stable proteins having a chaperone-like activity such as in the claimed invention have been identified in diverse species, from poplar, tomato, pine, rice, corn, grains and yeast, and that many of these proteins have demonstrated common structural characteristics, likely associated with their biological activity.

The following Tables provides a summary of the similar sequence/structural and functional characteristics of stress-related, boiling and detergent stable proteins having a chaperone-like activity, as disclosed in the instant specification and subsequent publications.

TABLE I

Sequence/Structure similarity	PLANT OF ORIGIN: Tomato	Pine	Rice	Corn	Arabidopsis	Aspen (SEQ ID NO: 2)
Antigenic Cross-reactivity	+ Figs. 14c and 14d	+ Figs. 14a and 14b	+ Page 76, line 4 and 5	+ Page 76, line 4 and 5	+ (See Declaration)	+
Sequence homology (positives) to SEQ ID NO: 2	65% SEQ ID NO: 16		73% SEQ ID NO: 11	71% SEQ ID NO: 10	68% SEQ ID NO: 13	100%
3D structure (X-Ray crystallography, EM) (Secondary structure)					Four β-strands and three α-helixes in the hydrophilic region (*Dgany et al*)	Four β-strands and three α-helixes in the hydrophilic region (*Dgany et al*)
Quaternary Structure	Oligomeric formation Figs. 14c and 14d	Oligomeric formation Figs. 14a and 14b			Dimer formation	Dimer and Oligomer formation (Fig. 8), Ring-like structure (Fig. 10)
Conserved sequence Homology (HVFESTFES, corresp. aa 61-75 of SEQ ID NO:2)	+		+	+	+	+

TABLE II

Functional similarity	PLANT OF ORIGIN: Tomato	Pine	Rice	Corn	Arabidopsis	Aspen (SEQ ID NO: 2)
Isolation of Boiling, Detergent and Protease –Stable proteins	+ Figs. 14c and 14d, Table 1	+ Figs. 14a and 14b	+ Table 1	+ Table 1	+ Table 1	+
Chaperone-like activity	+ Fig. 9 Table 1	+ Fig. 9	+ Table 1	+ Table 1	+ Table 1	+
Stress-Related (and/or stress inducible)	+ Figs. 14c and 14d, Table 1	+ Figs. 14a and 14b			At3g17210-deficient (KO) plants become stress-susceptible (See Declaration)	+ Fig. 21a-21c

Table I summarizes the sequence and structural similarity between boiling and detergent-stable proteins from Aspen (SEQ ID NO:2) and other, diverse plant species (Tomato, Pine, Rice, Corn and Arabidopsis) having at least 65% amino acid homology to SEQ ID NO: 2.

Comparison of the sequence/structure data illustrates that according to a variety of important physical criteria, significant similarity exists between the claimed proteins. Comparison of antigenic cross reactivity indicates that antibodies

raised against natural and recombinant Aspen boiling and detergent-stable protein (SEQ ID NO:2) detect all of the stable proteins isolated from Tomato, Pine, Rice and Corn on Western blot. Further, antibodies raised against boiling and detergent-stable protein from Arabidopsis (SEQ ID NO: 13) detected the Aspen boiling and detergent-stable protein (SEQ ID NO:2) (see Declaration, Appendix Fig. 1). Thus, boiling and detergent-stable proteins from diverse species, having at least 65% sequence homology, exhibit shared epitopes, thus demonstrating shared sequence/structure characteristics.

Further, boiling and detergent-stable proteins from diverse species show a similar tendency to formation of quaternary structures. As shown in the instant specification, the boiling and detergent-stable proteins from Tomato and Pine (Figs. 14 a-d), as well as Aspen (Figs. 2a-c), when separated on SDS-PAGE, retain discrete higher molecular weight forms detectable by anti-Aspen boiling and detergent-stable protein antibodies, even under severe denaturing conditions (12-17 % SDS and boiling) (indicating dimer and oligomer formation). Further, as noted hereinabove, stable dimer formation characterizes the Arabidopsis and Aspen boiling and detergent-stable proteins.

Regarding conserved domains, the instant specification discloses that boiling and detergent-stable proteins of diverse species share a major conserved region of HVFESTFES, and minor conserved regions of VKH and KSF (see Fig. 12, page 29, lines 23-29 and page 72, lines 10-16 of the instant specification). Structural analysis of the Aspen (SEQ ID NO: 2) and Arabidopsis (SEQ ID NO: 13) boiling and detergent-stable proteins indicate that this region is critical for stable dimer formation (Dgany et al, page 51522).

Thus, boiling and detergent-stable proteins from diverse species having at least 65% sequence homology exhibit shared epitopes, and share at least one conserved region of amino acid homology important in the characteristic stable dimer formation and thermostable properties of the boiling and detergent-stable proteins.

Table II *supra* summarizes the functional similarity between boiling and detergent-stable proteins from Aspen (SEQ ID NO:2) and other, diverse plant species (Tomato, Pine, Rice, Corn and Arabidopsis) having at least 65% amino acid

homology to SEQ ID NO: 2. Table 1 of the instant specification (page 80) illustrates the correlation between boiling and protease resistance and chaperone activity. Boiling and protease resistant fractions of Arabidopsis, Tomato, Corn and Rice all retained chaperone like activity in the HRP assay. Significant chaperone-like activity, equal to or greater than that of Aspen, was also detected in boiling-stable fractions of Pine and Tomato using the CS-thermostability assay (Figure 9 and page 75, lines 12-22 of the instant specification). Thus, boiling and detergent-stability in proteins from diverse species having at least 65% sequence homology to SEQ ID NO: 2 is consistently correlated with the presence of chaperone-like activity, as determined by the thermostability assays of the CS dimer and HRP monomer.

Aspen boiling and detergent stable protein (SEQ ID NO:2) of the present invention was originally identified in water-stressed Aspen shoots (Pelah, et al. 1995, of record). While reducing the present invention to practice, it was further uncovered that boiling stable extracts of water-, salt- and temperature-stressed Tomato and Pine contain significantly greater amounts of immune-cross-reactive boiling and detergent-stable proteins than unstressed plants (Figures 14a- 14d). Yet further, while reducing the present invention to practice, it was uncovered that overexpression (under control of a constitutive promoter) of SEQ ID NO:2 in transgenic Aspen plants provided significant protection, and hastened recovery from experimental salt stress (Figures 21a-c of the instant specification).

Thus, as summarized in Table II, the claimed boiling and detergent-stable proteins from Aspen (SEQ ID NO:2) and other, diverse plant species (Tomato, Pine, Rice, Corn and Arabidopsis) having at least 65% amino acid homology to SEQ ID NO: 2, immune-cross reactivity, and sharing a conserved region of sequence homology also share similar functional characteristics, specifically, chaperone-like activity and stress-relatedness.

Taken together, these data strongly indicate a significant connection between the common structural/sequence characteristics, the similar stability and chaperone like activity, and the stress-related function exhibited by the claimed boiling- and detergent-stable proteins of diverse species.

In view of the data presented hereinabove, Applicant is of the strong opinion

that claimed boiling and detergent stable proteins having a chaperone-like activity and at least 65% homology to SEQ ID NO: 2 of the present invention are representative species of a genus of proteins well defined by structural and sequence criteria imparting similar functional properties.

The Examiner has rejected claims 133-134, 138-139 and 143-144 under 35 U.S.C. § 112 1st paragraph, for failing to comply with the written description requirement. The Examiner's rejections are respectfully traversed. Claims 132-146 have now been amended.

The Examiner has asserted the instant specification fails to adequately describe a "polypeptide of instant SEQ ID NO: 2 or homologs thereof, that have chaperone-like activity", asserting that "nowhere in the specification is it described which amino acids are essential or critical for...functionality" and that "the concept(ion) is not achieved until reduction to practice has occurred". Further, the Examiner has implied that the instant specification relies merely on "a statement that it [the claimed composition] is part of the invention". Applicant strongly disagrees.

Tables I and II *supra* summarize the structure-function relationships for the claimed stress-resistant, chaperone-like proteins homologous to SEQ ID NO:2, as disclosed in the instant specification. Clearly, the claimed polypeptides are described and identified in the instant specification with respect to the conserved domains, antigenic cross-reactivity, the similar stability and chaperone like activity, and the stress-related function exhibited by boiling- and detergent-stable proteins of diverse species.

Yet further, the Examiner has rejected claims 132, 135-137, 140-142, 145-146 and 171-176 under 35 USC § 112, 1st paragraph, for referring to a protein only by the function. Claims 173-176 have now been canceled, rendering moot the Examiner's rejection thereof. Claims 132-146 and 171-172 have now been amended to include the limitations of a polypeptide having at least 65% homology to SEQ ID NO: 2, and at least one conserved amino acid sequence in at least one region corresponding to amino acids 9-11, 47-49 and/or 61-75, of SEQ ID NO:2 (see *infra*), thereby overcoming the Examiner's rejection thereof.

The abovementioned notwithstanding, and in order to expedite prosecution in this case, Applicant has elected to amend independent claim 132 to include the limitations of:

> "A fusion protein comprising a boiling stable polypeptide fused to an additional polypeptide" and
> "...at least 65% homologous to SEQ ID NO:2, said boiling and detergent stable protein having a chaperone-like activity, said protein having at least one conserved amino acid sequence in at least one region corresponding to amino acids 9-11, 47-49 and/or 61-75, of SEQ ID NO:2 as determined using a Best Fit software of the Wisconsin sequence analysis package, utilizing the Smith and Waterman algorithm, wherein gap creation penalty equals 8 and gap extension penalty equals 2"

Thus now amended claims 132-136 and 171-172 refer to a defined group of fusion proteins comprising a boiling stable protein having similar function (boiling- and detergent stability, and chaperone-like activity) and similar structural characteristics (at least 65% homology to SEQ ID NO: 2 and conserved sequence homology), and an additional polypeptide. Support for such an amendment is found in claim 134, previously presented claim 171, in Figure 12, and on page 71, line 25 to page 72, line 12 of the instant specification:

> "Nucleotide sequence analysis of the cDNA (Wisconsin Package Version 9.1, Genetics Computer Group-GCG, Madison WI.) indicated that the SP1 cDNA encodes a 12.368 kDa polypeptide with a calculated pI of 4.87. Analysis of the open reading frame revealed that this polypeptide lacks Cystein residues, is low in Tryptophan residues (0.9 %), and is rich in Leucine (13.8 %), Threonine (9.2 %), Alanine (8.3 %), Glutamic (7.4%) and Serine (7.4%) residues. No homology was detected with any known protein sequences in the SWISS-PROT protein bank. Coding sequences exhibiting sequence homology with SP1 from various evolutionary distant plant species were identified using the EST database (Plurality = 10.0; Threshold = 4; Average Weight = 1.00; Average Match = 2.91; Average Mismatch = -2.00). 25 sequences with significant homology (E value below 0.5) were identified (3 in Arabidopsis, 2 in maize, 1 in potato, 2 in rice, 1 in sorghum, 7 in soybean, 2 in tomato and 7 in wheat, see Figure 12 and SEQ ID NOs:7-32, Consensus Sequence - SEQ ID NO:33). The putative peptide sequences

were aligned and compared with the peptide sequence of SP1 (SEQ ID NO:2), revealing a few conserved consensus sequences: "HAFESTFES" (61-75, SEQ ID NO:36), "VKH" (9-11, SEQ ID NO:37) and "KSF" (47-49, SEQ ID NO:38)."

In light of the arguments and amendments brought hereinabove, it is Applicant's strong opinion that one of ordinary skill in the art, in possession of the teachings of the instant specification, would be capable of readily identifying and using the claimed boiling- and detergent stable proteins, according to the structural/sequence and functional properties, as recited in amended claim 132, using art-recognized methods and without need for undue experimentation. Withdrawal of the 35 USC 112 1st paragraph rejections is respectfully requested.

Claims Rejections: 35 U.S.C. § 112 2nd Paragraph

The Examiner has rejected claims 134, 139, 144 and 171-176 under 35 U.S.C. 112 2nd paragraph for failing to point out and distinctly claim the subject matter which is regarded as the invention. The Examiner's rejections are respectfully traversed. Claims 173-176 have now been canceled, rendering moot the Examiner's rejection thereof. Claims 132-146 and 171-172 have now been amended.

Claims 132- 136 have now been amended and recite the term "identical" in place of "homologous"

Regarding claims 171-176, the Examiner has asserted that the claims are unclear because they do not identify the additional polypeptide. Applicant disagrees.

It will be appreciated that the fusion proteins of now amended claim 132, and claims dependent therefrom, comprise a polypeptide defined by function, sequence homology and the presence of conserved domains, fused to an additional polypeptide. The additional polypeptide can be any peptide, fused to the stable protein of the invention by one of many methods known in the art, and detailed in the instant specification (see pages 54 to 56). such as growth factors, glycosil-hydrolases, peroxidases, transferases, kinases, phosphatases, sulfatases, nucleic-acid-modifying enzymes (ligases, restriction enzymes, reverse-transcriptase, nucleic ccid polymerases).

The abovementioned notwithstanding, claim 132 now includes the limitations of "...said peptide being selected from the group consisting of an aggregating protein, a messenger protein, a messenger protein receptor protein, a protein of an agent of an infectious disease, a protein of an agent of a non-infectious disease, an interferon protein, an interferon receptor protein, a protein antigen, a growth factor protein, an enzyme protein and a heterologous protein of transformed cells". Support for such a limitation, and the limitations of amended claims 138-172 can be found throughout the instant specification, for example, page 54, lines 1-20:

> "The following provides a non-exhaustive list of proteins having known genes which can be fused to a stable protein of the invention: proteins having medicinal properties: aggregating proteins such as beta amyloid, messenger proteins such as the cytokines IL-1 and IL-7, and their receptor proteins, proteins of agents of infectious diseases, such as bacterial exported proteins from *pneumococci*, *streptococci* and other pathogenic strains, proteins from pathogenic viruses such as hepatitis B and transmissible gastroenteritis, and from protozoa and helminths in parasitic infections; non-infectious diseases, such as poorly antigenic autologous tumor cell proteins or any of their epitopes, interferons and their receptor proteins in the case of autoimmune diseases, proteins useful in research, including protein or polypeptide reagents for immuno-assays such as insulin, gastrin, opiods, growth factors, calcitonin, malarial and other protozoan blood-stage antigens, enzymes such as peroxidase and heat or detergent labile biologically active proteins, including enzymes and proteins useful in commercial applications, e.g., proteases, glycosil-hydrolases and lipases, heterologous proteins aggregating in transformed cells or their culture media such as growth factors, glycosil-hydrolases, peroxidases, transferases, kinases, phosphatases, sulfatases, nucleic-acid-modifying enzymes (ligases, restriction enzymes, reverse-transcriptase, nucleic acid polymerases)."

And in Examples, pages 72-74. Support for at least 65%, at least 75%, at least 80%, at least 85% and at least 95% homology to SEQ ID NO: 2 is found throughout the instant specification, for example, page 34, lines 14-22.

Thus, Applicant respectfully requests withdrawal of the 112, 2nd paragraph rejections.

Claims Rejections: 35 U.S.C. § 102 Rejections: Wang et al. (2001 ISHS Acta Horticulture 560), Leung et al (1996, Cell Stress and Chaperones, 1) and Wang et al (US 20050074763)

The Examiner has rejected claims 132-146 and 171 as lacking novelty under 35 U.S.C. 102(a). Claims 132, 135-137, 140-142 and 145-146 have been rejected as anticipated by Wang et al (2001), claims 142 and 145-146 have been rejected as anticipated by Leung et al. (1996) and claims 171-176 have been rejected as anticipated by Wang (US 20050074763). The Examiner's rejections are respectfully traversed. Claims 173-176 have now been canceled, rendering moot the Examiner's rejection thereof. Claims 132-146 and 171-172 have now been amended.

Claim 132 has now been amended to include the limitations of previously presented claim 171, and now reads on fusion proteins comprising a boiling stable protein having similar function (boiling- and detergent stability, and chaperone-like activity) and similar structural characteristics (at least 65% homology to SEQ ID NO: 2 and conserved sequence homology), and an additional polypeptide. Inasmuch as Wang et al (2001) and Leung et al (1996) are silent with regard to a fusion protein as claimed, Applicant believes to have overcome the Examiner's 35 USC 102(a) rejections on the basis of Wang et al (2001) and Leung et al (1996).

Regarding the rejections on the basis of Wang et al (US 20050074763), claim 132 now includes the limitations of previously presented claim 171, reading on fusion proteins having homology to SEQ ID NO: 2 and an additional polypeptide. Applicant wishes to point out that, as detailed *supra*, the instant specification includes all of the disclosures of both PCT IL03/00723 and PCT IL02/00174, and as such includes the entire disclosure of US 20050074763. A petition for unintentional delayed claim of priority, according to 37 USC 1.78(a)(3) is being filed, along with requisite fees, effectively determining identical priority for the present application and the cited publication. In view of this, Applicant respectfully requests withdrawal of the 102(a) rejection on the basis of Wang et al (US 20050074763).

In view of the above amendments and remarks it is respectfully submitted that amended claims 132-146 and 171-172 are now in condition for allowance. Prompt notice of allowance is respectfully and earnestly solicited.

Respectfully submitted,

Martin D. Moynihan
Registration No. 40,338

Date: February 28, 2008

Encls:

- Petition and Fee for One (1) Month Extension
- Cited reference Dgany et al
- Petition to Add Priority Application

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The Structural Basis of the Thermostability of SP1, a Novel Plant (*Populus tremula*) Boiling Stable Protein*

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Or Dgany‡§, Ana Gonzalez¶, Oshrat Sofer‡, Wangxia Wang§, Gennady Zolotnitsky‖, Amnon Wolf, Yuval Shoham‖, Arie Altman§, Sharon G. Wolf‡‡, Oded Shoseyov§, and Orna Almog‡§§**

*From the ‡Department of Clinical Biochemistry, Faculty of Health Sciences, Ben-Gurion University, Beer-Sheva 84105, Israel, the §Robert H. Smith Institute of Plant Sciences and Genetics in Agriculture, The Hebrew University of Jerusalem, P.O. Box 12, Rehovot 76100, Israel, the ¶Stanford Synchrotron Radiation Laboratory, Menlo Park, California 94025, the ‖Department of Biotechnology and Food Engineering, Institute of Catalysis Science and Technology, Technion-Israel Institute of Technology, Haifa 32000, Israel, **Fulcrum SP Ltd., P.O. Box 3206 Herzliya 46104, Israel, and the ‡‡Electron Microscopy Unit, Weizmann Institute of Science, Rehovot 76100, Israel*

We previously reported on a new boiling stable protein isolated from aspen plants (*Populus tremula*), which we named SP1. SP1 is a stress-related protein with no significant sequence homology to other stress-related proteins. It is a 108-amino-acid hydrophilic polypeptide with a molecular mass of 12.4 kDa (Wang, W. X., Pelah, D., Alergand, T., Shoseyov, O., and Altman, A. (2002) *Plant Physiol.* 130, 865–875) and is found in an oligomeric form. Preliminary electron microscopy studies and matrix-assisted laser desorption ionization time-of-flight mass spectrometry experiments showed that SP1 is a dodecamer composed of two stacking hexamers. We performed a SDS-PAGE analysis, a differential scanning calorimetric study, and crystal structure determination to further characterize SP1. SDS-PAGE indicated a spontaneous assembly of SP1 to one stable oligomeric form, a dodecamer. Differential scanning calorimetric showed that SP1 has high thermostability *i.e.* T_m of 107 °C (at pH 7.8). The crystal structure of SP1 was initially determined to 2.4 Å resolution by multi-wavelength anomalous dispersion method from a crystal belonging to the space group I422. The phases were extended to 1.8 Å resolution using data from a different crystal form (P21). The final refined molecule includes 106 of the 108 residues and 132 water molecules (on average for each chain). The *R*-free is 20.1%. The crystal structure indicated that the SP1 molecule has a ferredoxin-like fold. Strong interactions between each two molecules create a stable dimer. Six dimers associate to form a ring-like-shaped dodecamer strongly resembling the particle visualized in the electron microscopy studies. No structural similarity was found between the crystal structure of SP1 and the crystal structure of other stress-related proteins such as small heat shock proteins, whose structure has been already determined. This structural study further supports our previous report that SP1 may represent a new family of stress-related proteins with high thermostability and oligomerization.

Stable protein 1 (SP1),[1] which we previously isolated from aspen plants (*Populus tremula*) (2–4), is a boiling stable, stress-responsive protein with no significant sequence homology to other stress-related proteins (5–7). SP1 cDNA encodes a 12.4-kDa hydrophilic polypeptide having no cysteine or potential glycosylation sites (1). Amino acid analysis and the N-terminal sequence of SP1 revealed that SP1 is a homooligomeric protein composed of 12 subunits that are tightly bound to each other even under extreme conditions. Similar to native SP1, recombinant SP1 produced in both *Escherichia coli* and *Pichia pastoris* assembles spontaneously to a multimeric stable complex (8).

Environmental stresses, such as drought, salinity, or heat, induce specific genes in higher plants. Plants inherently possess various molecular-biochemical mechanisms by which they can cope with such stresses (6, 9, 10). One of the mechanisms that may confer such tolerance is the activation of a large set of genes that leads to the accumulation of specific proteins in the cells. Several families of proteins have been identified as stress-induced proteins (8). The small heat shock protein (Hsps) family is the major type of stress-induced proteins and is believed to exert cellular protection during stress (5, 11). Hsps are molecular chaperones that are characterized by their ability to selectively recognize and bind to misfolded proteins. They possess high thermostability and create oligomers spontaneously.

Attempts to identify the factors responsible for the high thermostability of proteins have included sequence and structural comparisons of homologous proteins from mesophile, thermophile, and hyperthermophile organisms (12–16). These comparisons have shown that proteins isolated from thermophile organisms have a better internal packing of non-polar groups, more hydrogen bonds, and more ion pairs than homologous proteins isolated from mesophiles

Here we performed a thermostability study of SP1 using the differential scanning calorimetry (DSC) method, and we also determined the crystal structure of SP1 at 1.8 Å resolution. The DSC study showed a T_m of 107 °C for SP1, which supports our

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The atomic coordinates and structure factors (code 1SI9 and 1TR0) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

§§ To whom correspondence should be addressed. Tel.: 972-8-647-9930; Fax: 972-8-647-9931; E-mail: almogo@bgu.ac.il.

[1] The abbreviations used are: SP1, stable protein 1; DSC, differential scanning calorimetry; EM, electron microscopy; sHsp, small Hsp.

TABLE I
Data processing and merging statistics

MAD data set from Se-Met I422 crystal						
Lambda 0.979 Å						
Resolution (Å)			40.83 - 2.4			
Number of reflections			93175			
Number of unique reflections			15619			
	R-sym	R-anom	I/sigma(I)	Completeness (%)	Multiplicity	Anomalous completeness (%)
Overall	0.095	0.067	6.5	100	6.0	99.7
Last resolution bin (2.57-2.4 Å)	0.521	0.289	1.4	99.9	5.1	99.3
Lambda 0.905 Å						
Resolution (Å)			40.83 - 2.4			
Number of reflections			87157			
Number of unique reflections			15570			
	R-sym	R-anom	I/sigma(I)	Completeness (%)	Multiplicity	Anomalous completeness (%)
Overall	0.096	0.058	6.3	99.7	5.6	97.8
Last resolution bin (2.57-2.4 Å)	0.487	0.299	1.5	99.0	4.3	92.0
Lambda 0.980 Å						
Resolution (Å)			25.410 - 2.4			
Number of reflections			90275			
Number of unique reflections			15590			
	R-sym	R-anom	I/sigma(I)	Completeness (%)	Multiplicity	Anomalous completeness (%)
Overall	0.092	0.056	6.9	99.8	5.8	98.8
Last resolution bin (2.57-2.4 Å)	0.541	0.300	1.3	99.2	4.6	95.4
Native data set from I422 crystal						
Lambda 0.954 Å						
Resolution (Å)			40.8-2.0			
Number of reflections			125502			
Number of unique reflections			18279			
		R-sym	I/sigmal	Completeness (%)	Multiplicity	
Overall		0.078	6.3	99.9	6.9	
Last resolution bin (2.45-2.27 Å)		0.340	2.4	100.0	6.9	
Native data set from $P2_1$ crystal						
Lambda 0.954 Å						
Resolution (Å)			48.5 - 1.8			
Number of reflections			1219831			
Number of unique reflections			35953			
		R-sym	I/sigmal	Completeness (%)	Multiplicity	
Overall		0.087	8.6	92.5	1.9	
Last resolution bin (1.9-1.8 Å)		0.447	2.8	88.4	1.9	

previous finding that the SP1 is a boiling stable protein. The crystal structure of SP1 was refined to a crystallographic *R*-factor of 16.4%. The SP1 monomer has a ferredoxin-like fold or an α/β structure of four β-strands and three α-helices. Monomers of SP1 assemble into a dimer via their hydrophobic interface. The hydrophobic inner core and dimer interface probably contribute to the enhanced thermostability of SP1 as well as its oligomerization. Sequence and structural comparisons between SP1 and other stress-related proteins, such as small Hsp (sHsp), suggest that SP1 represents a new class of stress-related proteins.

EXPERIMENTAL PROCEDURES

SDS-PAGE of SP1

SP1 samples (wild type and recombinant) were purified as reported earlier (1). Wild-type SP1 samples (10 mg/ml) were subjected to SDS-PAGE using sample buffer (SB, containing 2% SDS) with or without boiling treatment (10 min). The low molecular weight band (which corresponds to the monomeric form of SP1) was electro-eluted from the SDS-PAGE and dialyzed overnight against 25 mM Tris, 200 mM glycine, and 0.025% SDS. This low molecular band protein sample was subjected again to SDS-PAGE using SB with or without boiling treatment.

DSC Study

Solutions of recombinant SP1 (24 μmol in a sodium phosphate buffer, pH 7.8) were scanned with a Microcal VP-DSC-ER calorimeter (Microcal Inc., Northampton, MA) at a scan rate of 60 degree/h^{-1}. The data was analyzed using the 5.0 software supplied by the DSC manufacturer. The data was analyzed using Origin 5.0 software (MicroCal).

Crystallization

Crystallization experiments were carried out at room temperature using the hanging drop vapor-diffusion method with VDX-24 tissue culture plates (Hampton HB3–140). Droplets of the protein solution containing 7 mg/ml protein in 50 mM Tris, pH 7.5, 100 mM NaCl, 20 mM $CaCl_2$ were mixed with an equal volume of reservoir solution of 20% polyethylene glycol 3000, 0.1 M HEPES, pH 7.5, and 0.2 M NaCl. Crystals appeared within a few days and were grown to their maximal size in 2 weeks. These crystals belong to the space group I422 with unit cell $a = b = 90.50$ Å, $c = 186.30$ Å (17). New crystals with different morphology appeared within the same droplet after 3 weeks. These new crystals belong to the P21 space group with unit cell of $a = 97.03$ Å, $b = 94.75$ Å, $c = 168.03$ Å, $\beta = 90.11°$. Selenium-methionine crystals were also crystallized in the I422 space group with unit cell $a = b = 90.75$ Å, $c = 186.41$ Å.

X-ray Diffraction Data Collection and Processing

All the data used for solving and refining the structure were collected on the SSRL wiggler beamline 9-2, using a 2 × 2 area detector systems corporation charge-coupled device detector. Both the native and selenium-methionine crystals were transferred from the mother liquor to the cryoprotectant (13% glycerol and 87% mother liquor). After a few seconds, the crystals were scooped up in a cryoloop and exposed to a cold nitrogen stream (Oxford Instruments), where they were rapidly frozen to ~100 K. Multiwavelength anomalous diffraction data were collected on selenium-methionine crystals belonging to the space group I422. The data were collected at three wavelengths around the selenium absorption edge (see Table I). Native data was collected from the tetragonal and monoclinic crystals.

All the data (three-wavelength multiwavelength anomalous diffraction and two native data sets) were integrated with the MOSFLM program package (18). The data were scaled and merged using SCALA (19, 20). The structure factor amplitudes were calculated using the TRUNCATE program (19, 21). Data statistics derived from data processing are shown in Table I.

Structure Solution

Tetragonal Structure—The phases for the selenium-methionine data were calculated with SOLVE-RESOLVE. Six selenium sites were located for three selenium-methionine found in SP1 sequence. Because the Mathews coefficient (22) was very high for two copies of the molecule in the asymmetric unit, it was assumed that there were three molecules and that the N-terminal methionine was disordered. A trimer in the asymmetric unit resulted in a Mathews coefficient of 2.8 for a solvent content of 56% of the unit cell volume. The mean experimental figure of merit was 0.34–2.4 Å. The non-proper non-crystallographic symmetry operations relating the monomers could be estimated from the selenium sites. The CCP4 program DM (23) was used for refining the non-crystallographic symmetry operations and averaging and extending the phases to the native data set resolution. The model was built into the map calculated with these modified phases.

TABLE II
Summary of the refinement for the $P2_1$ and I422 models

I422 model	
Refined residues	106
Solvent atoms	441
Glycerol	1
Ramachandran most favored region	94–91
Ramachandran allowed region	5–2
Ramachandran generously allowed region	0
Ramachandran disallowed region	0
R-factor (all reflections, work + free set	0.209
R-factor (highest resolution bin)	0.320
Reflections in free set	910
R-factor (free set)	0.258
R-factor (free set in highest resolution bin)	0.341
Correlation coefficient (all reflections)	0.949
Correlation coefficient (free set)	0.931
Overall coordinate e.s.u.[a]	0.232
Overall B e.s.u.[a]	10.259
$P2_1$ model	
Refined residues	106
Solvent atoms	3179
Glycerol	1
Ramachandran most favored region	91–94
Ramachandran allowed region	2–5
Ramachandran generously allowed region	0
Ramachandran disallowed region	0
R-factor (all reflections, work + free set)	0.164
R-factor (highest resolution bin)	0.231
Reflections in free set	13491
R-factor (free set)	0.202
R-factor (free set in highest resolution bin)	0.250
Correlation coefficient (all reflections)	0.963
Correlation coefficient (free set)	0.940
Overall coordinate e.s.u.[a]	0.079
Overall B e.s.u.[a]	2.623

[a] Based on maximum likelihood target $R = \Sigma (|F_o| - |F_c|)/\Sigma|F_o|$. e.s.u., estimated standard uncertainties.

Monoclinic Structure—The structure in the space group P21 was solved by molecular replacement using the structure of the I422 crystal form. Attempts to solve the structure using one to three copies of the molecule refined in the I422 space group as a search model failed. Previous EM studies showed that SP1 has a ring-like shape with a central cavity. EM studies also revealed oligomers possessing significant homogeneity in shape and size, which clearly demonstrated a 6-fold symmetry of the SP1 particle along the axis of the ring.[2] It was then noticed that the dodecamer formed by the three molecules in the I422 asymmetric unit and nine additional molecules related to the first three by the I422 symmetry operations $-x, 1 - y, z; y - 0.5, x + 0.5, -z + 0.5$; and $-y + 0.5, -x + 0.5, -z + 0.5$ had a pseudo 6-fold axis and a shape and size resembling the particles in the EM studies. Using this dodecamer as a search model yielded a clear solution for the P21 structure using the program MOLREP (19, 24). Two dodecamers were found in the P21 asymmetric unit (Matthews coefficient 2.7).

Model Refinement—Both the tetragonal and monoclinic models were refined with the program REFMAC (Collaborative Computational Project, 1994) using a maximum likelihood target (24). The geometry was restrained to the standard Engh and Huber values (34), using the maximum likelihood target. The non-crystallographic symmetry and, in the case of the I422 structure, the multiwavelength anomalous diffraction experimental phases, were also used as additional restraints. Restrained refinement was preceded by translation, liberation, and screw rotation parameters refinement (25). Hydrogen atoms were generated before refinement in their riding positions and used for geometry and structure factor calculations. Automatic water molecule searching was carried out using automated refinement of protein crystallography (26).

After refinement, the fit of the model to the electron density was

[2] O. Dgany, A. Gonzalez, O. Sofer, W. Wang, G. Zolotnitsky, A. Wolf, Y. Shoham, A. Altman, S. G. Wolf, O. Shoseyov, and O. Almog, unpublished data.

FIG. 1. **SDS-PAGE analysis of SP1 and electroeluted SP1.** Wild-type SP1 without boiling treatment is shown in *lane 1*. Wild-type SP1 boiled in the presence of 2% SDS is shown in *lane 2*. The low molecular band was electroeluted and resubjected to SDS-PAGE. The low molecular band reassembled to a high oligomeric form (*lane 3*) and dissociated to the same low molecular weight band after another boiling treatment in the presence of 2% SDS (*lane 4*).

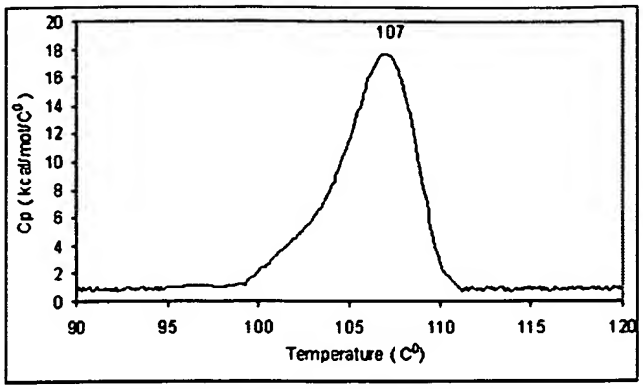

FIG. 2. **DSC scan of SP1 indicating T_m for SP1 of 107 °C, which further supports evidence that SP1 is a boiling stable protein.**

inspected using difference Fourier density maps $2mF_o - DF_c$ and $mF_o - DF_c$, where F_o and F_c are the scaled observed and calculated structure factors, m is the figure of merit, and D is an estimate of the error in the partial structure from coordinate errors (27). These coefficients were calculated by REFMAC. The results of the refinement and the stereochemical analysis of the model, carried out with PROCHECK (28), are summarized in Table II. The final refined models have been deposited in the PDB with the code 1SI9 for the I422 crystal form and the code 1TR0 for the P21 crystal form.

RESULTS

SDS-PAGE Analysis and DSC Study of SP1

Fig. 1 shows the results of a SDS-PAGE analysis of wild-type SP1 isolated from aspen plants. Fig. 1, *lane 1* shows wild-type SP1 without boiling treatment as one high molecular band, which may correspond to the SP1 dodecamer. After boiling for 10 min the SP1 dodecamer dissociates to a lower molecular weight band which may correspond to the monomeric form of SP1 (Fig. 1, *lane 2*). This monomeric form of SP1 was electroeluted and subjected again to SDS-PAGE. Fig. 1, *lane 3* shows that the electro-eluted SP1 spontaneously reassembled to the same high oligomeric form, and upon boiling it dissociated to its low oligomeric form. The SP1 dodecamer dissociated only upon boiling in the presence of 2% SDS; however it reassembled spontaneously to a dodecamer. Fig. 2 shows a DSC study of SP1 resulting in a T_m of 107 °C for SP1. These results further support our previous findings that SP1 is a boiling stable protein and that the high oligomeric form dissociate only upon boiling treatment in the presence of 2% SDS.

The Crystal Structure Description of SP1

The Monomer—The maximum average root mean square displacement between the monomers in the asymmetric unit is less than 0.20 Å (0.43 Å for the tetragonal model), which indicates an overall high similarity between all the SP1 molecules, although some side chains in surface loops could be modeled in different conformations in different molecules. The protein chain has an α and β folding with three α-helices, H1 (residues 23–39), H2a (residues 74–81), and H2b (residues 84–93), and a β-sheet formed by four antiparallel β-strands, B3 (residues 9–17), B1 (residues 45–50), B2 (residues 65–71), and B4 (residues 97–108). Secondary structure characterizations were carried out using the PROCHECK software package (28) (Fig. 3).

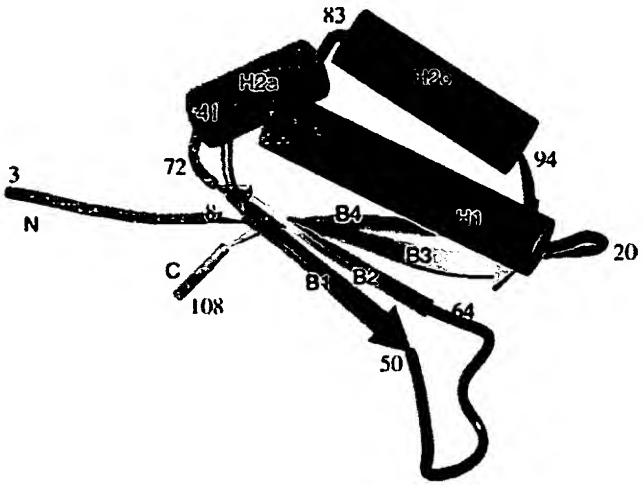

FIG. 3. **Crystal structure of SP1 monomer.** Secondary structure elements are shown as *red cylinders* (for α-helixes) and *yellow ribbons* (for β-strands). The SP1 monomer is composed of four β-strands (B1-B4) creating the hydrophobic region of the molecule, and three α-helixes (H1, H2a, H2b) form the hydrophilic region of the molecule.

The N-terminal segment points toward the solvent and is mobile as evidenced by the lack of interpretable electron density for the first two residues and the large temperature factors for Thr-3 and Lys-4. The long loop formed by residues 51–64 is largely unstructured. This loop projects away from the molecule and is involved in dimer contacts.

Helices H1 and H2 define an external convex surface with numerous hydrophilic and acidic side chains facing toward the solvent. The inner side of this surface and the opposing β-sheet enclose a hydrophobic central cavity rich in aromatic and hydrophobic residues (Fig. 4). Most of the phenylalanines in the SP1 molecule occupy this cavity (Phe-17, -46, -67, -71, -89, and -93). Trp-48 and Tyr-33, Tyr-63 and Tyr-80, together with the two histidines (His-11 and His-65), and Arg-100 block access of the solvent to the cavity. There are three waters buried in the central core of the monomer that also play a role in molecule stability. Wat125 forms three hydrogen bonds, one with Tyr-33 OH of helix H1 and the other two hydrogen bonds with Phe-67 O and Ser-69 O-γ of strand B2. Wat561 and Wat921 form an additional bridge between Tyr-33 O and Ser-69 O-γ. However, such non-hydrophobic interactions are rare in this hydrophobic pocket.

The Dimer—The dimer appears to be the smallest stable SP1 unit. The two molecules in the dimer are related by a 2-fold axis parallel to helix H1 and β-strands B3 and B4 (Fig. 5). The outer surface of the β-sheets of the two molecules forms a β barrel-

FIG. 4. **A stereoview of SP1 monomer showing the hydrophobic cavity inside each molecule.** The side chains (depicted in *purple*) correspond to aromatic residues (in *yellow*) to Val, Leu, Ile, and (in *green*) to cysteine residue.

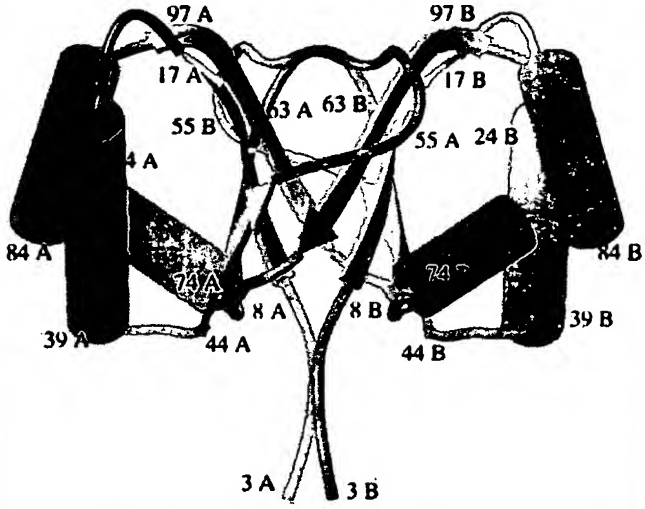

FIG. 5. **SP1 dimer composed of two SP1 molecules *A* and *B*.** In each chain residues are indicated along the crystal structure.

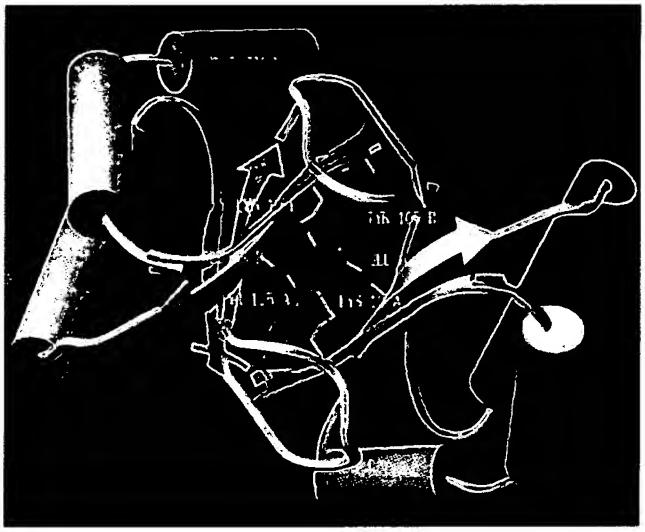

FIG. 6. **Dimer interactions involving Glu-68.** The distance between Glu-68 Oε-1 and Lys-10 N-ζ ranges from 2.7 to 2.8 Å (depending on the molecule). The distance between Glu-68 Oε-1 and Tyr OH ranges from 2.6 to 2.7 Å.

like structure, defining a central pore. At one end of the pore, the loop comprising the residues 51–64 bridges the gap between the two B4 strands in the dimer. The center of the pore is closed to the solvent by a barrier of hydrophobic side chains in B3 and B4 (Leu-14, Leu-101, Ile-103, and Tyr-105) and Tyr-63. The side chains of residues Asn-47, Ser-45, Thr-70, Leu-8, and Leu-107 occupy the surface at the end of the pore closer to the N termini.

Glu-68 is an important residue in creating stable dimers of SP1. Glu-68 side chain carboxylate forming hydrogen bonds to Lys-10 NZ and to the Tyr-105 side chain OH group of the opposite molecule (see Fig. 6). This residue is conserved in other proteins with the same fold as SP1. Water 5 mediates a dimeric contact bonding Phe-106 N and Thr-50 N in neighboring monomers. The complete list of direct dimer contacts is given in Table III.

The Dodecamer—The interdimer contacts predominantly involve hydrophilic side chains and charged groups or are mediated by water molecules. These contacts take place mainly along the B1, H1, and the N-terminal tails. The complete list of direct interdimeric contacts is given in Table IV. As a result of the interdimeric interactions six dimers create a ring-like structure around a pseudo 6-fold axis. The ring-like structure of the dodecamers has an outer radius of ~50 Å and an inner radius of ~15 Å (Fig. 7). The loop including residues 18–22 in each dimer protrudes toward the solvent, whereas the arms of the N-terminal are extending toward the inner part of the ring-like structure (Fig. 7). The 6-fold symmetry is broken, because the contacts between equivalent molecules in neighboring dimers are not identical.

In Fig. 8 the EM density map is superimposed on top of the crystal structure of a dodecamer. The high density regions of the EM map correspond to regions that are mainly α-helixes and are related to each other by the same pseudo 6-fold axis. The similarity of the dodecamer to the particles seen in EM studies (Fig. 8) and the fact that this arrangement is observed in different crystal space groups with no intrinsic 6-fold symmetry suggest strongly that the dodecamer is the biological unit active form of SP1.

TABLE III
Dimer contacts (molecules A and B)

Molecule A	Molecule B	Distance (Å)
Lys-10 N-ζ	Glu-68 Oε-1	3.0
Lys-10 N-ζ	Glu-68 Oε-2	2.9
Trp-48 N	Tyr-108 O	3.1
Trp-48 O	Tyr-108 N	2.8
Leu-52 N	Asp-104 O	3.0
Leu-59 O	Val-102 N	2.9
Arg-61 Nξ-1	Arg-100 O	2.9
Arg-61 Nζ	Arg-100 O	2.8
Glu-68 Oε-1	Lys-10 N-ζ	3.1
Glu-68 Oε-2	Lys-10 N-ζ	2.9
Arg-100 O	Arg-61 Nξ-1	2.8
Arg-100 O	Arg-61 N-ζ	2.8
Val-102 N	Leu-59 O	2.6
Asp-104 O	Leu-52 N	2.9
Tyr-105 OH	Glu-68 Oε-2	2.7
Tyr-108 N	Trp-48 O	2.8
Tyr-108 O	Trp-48 N	3.1

TABLE IV
Contacts between a dimer (molecules A and B) and its two new neighboring dimers (molecules C and D to its right and molecule K and L to its left)

Oxt, terminal oxygen.

	Neighboring dimer contact	Distance (Å)
Molecule A		
Thr-5 N	Lys-44 O (mol. C)	2.7
Lys-7 N-ζ	Phe-46 O (mol. C)	3.0
Lys-7 N-ζ	Asn-47 Oδ-1 (mol. C)	2.7
Lys-7 N-ζ	Tyr-108 O (mol. D)	3.1
Lys-7 N-ζ	Tyr-108 OXT (mol. D)	2.9
Tyr-108 O	Lys-7 N-ζ (mol. D)	3.3
Tyr-108 O	Lys-7 N-ζ (mol. D)	3.1
Asn-31 Oδ-1	Tyr-108 OH (mol. K)	2.7
Asn-31 Nδ-2	Thr-50 Oγ-1 (mol. L)	3.1
Thr-34 Oγ-1	Tyr-108 OH (mol. K)	3.1
Leu-37 O	Arg-4 Nξ-1 (mol. K)	3.2
Asp-38 Oδ-1	Ser-75 O-γ (mol. K)	3.0
Asn-47 Oδ-1	Lys-7 N-ζ (mol. K)	2.9
Thr-50 Oγ-1	Asn-31 N δ-2 (mol. L)	3.1
Molecule B		
Lys-18 O	Arg-23 N ζ-2 (mol. C)	3.0
Ile-21 O	Arg-23 N ζ-1 (mol. C)	2.8
Asn-31 Nδ-2	Thr-50 Oγ-1 (mol. C)	3.1
Asp-38 O δ-1	Ser-75 O-γ (mol. D)	2.9
Lys-44 O	Thr-5 N (mol. D)	2.7
Phe-46 O	Lys-7 N-ζ (mol. D)	2.8
Asn-47 Oδ-1	Lys-7 N-ζ (mol. D)	3.0
Thr-50 Oγ-1	Asn-31 Nδ-2 (mol. C)	3.0
Thr-3 Oγ-1	Lys-44 N-ζ (mol. L)	2.6
Thr-5 N	Lys-44 O (mol. L)	2.9
Thr-5 Oγ-1	Lys-44 O (mol. L)	3.3
Lys-7 N-ζ	Asn-47 Oδ-1 (mol. L)	2.9
Lys-7 N-ζ	Tyr-108 OXT (mol. K)	3.1
Lys-7 N-ζ	Tyr-108 O (mol. K)	3.2
Tyr-108 O	Lys-7 N-ζ (mol. K)	3.1
Tyr-108 OXT	Lys-7 N-ζ (mol. K)	2.9

The Interdodecamer Contacts—In the P21 space group the dodecamers are stacked in perpendicular interleaving layers (see Fig. 9, *a* and *b*). The interdodecamer interactions take place between dodecamers in perpendicular planes. There are water molecules that mediated hydrogen bonds between solvent-facing side chains in loop 16–22 of one dodecamer and the external surface of the dimer defined by a β-barrel in a neighboring dodecamer. There are also hydrophobic interactions that involve aliphatic side chains in the external loops. These contacts involve different residues for each SP1 molecule in the dodecamer, which explains the breakdown of the 6-fold symmetry in the crystal forms.

FIG. 7. **View of the dodecamer along the pseudo 6-fold axis.**

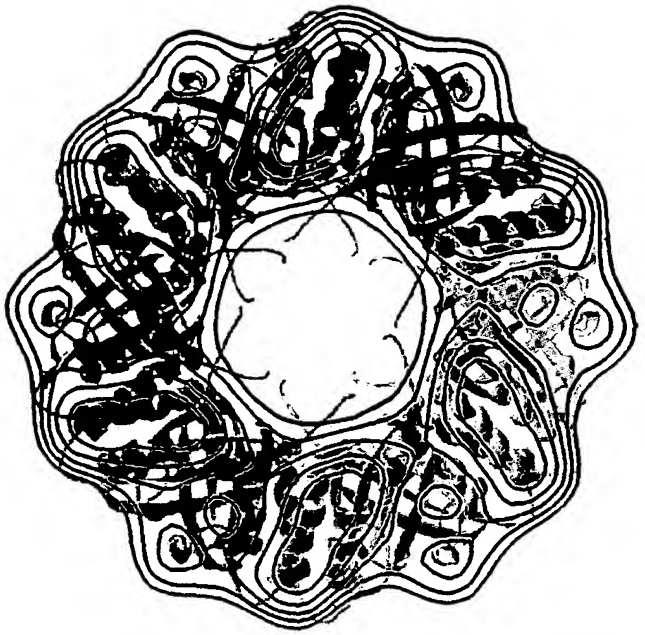

FIG. 8. **Superposition of the crystal structure of a dodecamer and the electron density map obtained from EM studies.**

DISCUSSION

The crystal structure of SP1 indicated strong hydrophobic interactions between two monomers that created a very stable dimer. With the exception of the N-terminal residues, the main chain of the protein was well defined into the electron density map, and no substantial differences, other than a better accuracy of the P21 model arising from the higher resolution of the diffraction and higher non-crystallographic symmetry, could be found between the molecules in the two different space groups.

Our results showed that SP1 belongs to the α+β protein class with ferredoxin-like fold composed of an α+β-sandwich with an antiparallel β-sheet. Two molecules of SP1 form a dimer. This dimer belongs to the superfamily of dimeric α+β-barrels in which the two β-sheets form a β-barrel. SP1 like other proteins in this family may assemble into higher order

a b

FIG. 9. **Crystal packing of the P21 crystal form viewed along the *xy* plane (*a*) and along the *z* axis (*b*).**

```
SP1          ..MATRTPKLXKHTLLTRFKDEITREQIDHYIHDYTWLLDLIPSHKSFHWGIDLGHESAE   58
At3G17210    GSHXEEARGPXKHVLLASFKDGVSPEKIEELIKGYAWLVELIEPXKAFHWGKDVSIENLH   60
ActVA-Orf6   ........AEXKDPRVGFVAVVTFPVDGPATQHKLVEIATGGVQEWIREVPGFLSATYHA   52

SP1          LNRGYTHAFESTFESKSGLQEYLDSAALAAFAEGFXPTLSQRLVIDYFLY            108
At3G17210    Q..YTHIFESTFEXKEAVAEYIAHPAHVEPATIFXGSLDKVLVIDYKPTSVSL         112
ActVA-Orf6   STDGTAVVNYAQWEXEQAYRVNFGADPRSAELREAIESSXPGLNGPPKAVFHTPRGAILPS  112
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FIG. 10. **Sequence alignment of SP1 with *A. thaliana* At3g17210, a stress-related protein, and ActVA-Orf6 monooxygenase reveals identical amino acids especially between SP1 and At3g17210 at residues 62–74 (GYTHXFESTFESK).**

oligomers. Proteins with the above mentioned dimeric $\alpha+\beta$-barrel folding include monooxygenase isolated from *Streptomyces coelicolor* (PDB entry 1LQ9) (ActVA-Orf6 (29)), TT1380 isolated from *Thermus thermophilus* (PDB entry 1IUJ), and *Arabidopsis thaliana* stress-related protein (PDB entry 1Q4R). In all of these cases, the B4 strand in one molecule creates hydrophobic interactions with the β-2 strand of the neighbor monomer, which results in a stable dimer. It is reasonable to assume that strong dimer interactions contribute to protein stability under extreme conditions. However, monooxygenase isolated from *S. coelicolor* was not reported to have high thermostability and was found to be active in its dimeric form.

The structural similarity of SP1 to TT1380 from *T. thermophilus* together with the apparent high T_m of SP1 (107 °C) implies that these proteins may use a ferredoxin-like fold as a scaffold for hyperthermostability and that they are evolutionally related. In addition to the thermostability of SP1 gained by creating dimers, it is possible that the assembly of dimers to dodecamers may also contribute to the high thermostability of SP1. Although the relationship between the degree of oligomerization and T_m is not yet clear (30), it is still possible that oligomerization contributes to the stability of SP1. Thermostable dodecamers as found for SP1 are rather rare for proteins with a ferredoxin-like fold. Most thermostable oligomers of this family of proteins are found in the range of monomers to tetramers (30, 31). Sequence comparisons of SP1 (Fig. 10) with proteins of ferredoxin-like fold such as *A. thaliana* At3g17210 stress-related protein and ActVA-Orf6 monooxygenase indicated a conserved region between SP1 and At3g17210 at residues 62–74 (GYTHXFESTFESK). This region corresponds to the region of the β-2 strand, which borders the helices 2 and 3 deep cavity, and it may have an important role in the function of these two proteins by serving as the active site in a way similar to that found for ActVA-Orf6 monooxygenase (17). It should be noted that the sequence similarity in this region found between monooxygenase and the above mentioned proteins is low. However this loop is the interdimer contact region as found for SP1, and it may also play a role in preserving the stability of SP1 (see Glu-68 role in dimer contacts).

Because SP1 may have some functional similarity to the small heat shock proteins family (sHsp) (*i.e.* it is stress-related and has high thermostability), we compared its structure to the only two sHsp crystal structures analyzed thus far, sHsp16.9 isolated from wheat (32) and sHsp16.5 isolated from *Methanococcus jannaschii* (33). These two sHsps have high amino acids sequence similarity between them and belong to the α-crystallin family. High structural similarity was found between the monomers of these two sHsps, and both monomers have a similar β-sandwich fold. In addition, both sHsps assemble to an oligomeric form. Hsp16.9 was found in an oligomeric form of a dodecamer composed of two stacking hexamers in which each hexamer is composed of three dimers, and the Hsp16.5 oligomer was found to be a hollow spherical complex composed of 24 units.

Nevertheless no amino acid sequence similarity exists between SP1 and these two sHsps. Radical differences also were found in the crystal structure of the SP1 and the two sHsps, indicating that SP1 does not belong to the conserved sHsps family. This may suggest that SP1 may be representing a different family of proteins with unique thermostability and a different function in stress protection.

Based on the present structural study of SP1 we can conclude that SP1 thermostability is a result of several factors. 1) SP1 has a ferredoxin-like fold, which was found in other studies to be a fold of several other thermostable proteins; 2) monomers of SP1 assemble into a stable dimer via a double hydrogen bond of residue Glu-68; 3) hydrophobic residues create interactions found in each inner core of the monomers and at the dimer interface; and 4) dimers form high oligomeric forms of SP1, such as a dodecamer, via additional interactions between the dimers.

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